

Brucella abortus RB51 and Hot Saline Extract from *Brucella ovis* as Antigens in a Complement Fixation Test Used To Detect Sheep Vaccinated with *Brucella abortus* RB51

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The efficacy of *Brucella abortus* RB51 and hot saline extract (HSE) from *Brucella ovis* as antigens in complement fixation (CF) tests was comparatively evaluated in detecting immune responses of sheep vaccinated with *B. abortus* strain RB51. For this study, four 5-month-old sheep were vaccinated subcutaneously with 5×10^9 CFU of RB51, and two sheep received saline. Serum samples collected at different times after vaccination were tested for the presence of antibodies to RB51 by a CF test with RB51 as antigen, previously deprived of anticomplementary activity, and with HSE antigen, which already used as the official antigen to detect *B. ovis*-infected sheep. The results showed that vaccinated sheep developed antibodies which reacted weakly against HSE antigen and these antibodies were detectable for 30 days after vaccination. However, antibodies to RB51 could be detected for a longer period after vaccination by using homologous RB51 antigen in CF tests. In fact, high titers were still present at 110 days postvaccination with RB51 antigen. Sera from sheep naturally infected with *B. ovis* also reacted to RB51 but gave lower titers than those detected by HSE antigen. As expected, all sera from RB51-vaccinated sheep remained negative when tested with standard S-type *Brucella* standard antigens.

Brucellosis is an economically important abortifacient disease of cattle and sheep that can also be transmitted to humans. Prevention of animal brucellosis is achieved by using live attenuated vaccines, such as S19 for cattle and Rev1 for sheep and goats. In many European countries including Italy, however, the routine use of vaccines against brucellosis is no longer allowed, and brucellosis eradication programs are based exclusively on serological screening of sheep and cattle herds to detect and remove infected animals (Decreto Ministeriale, 27 August 1994, no. 651, and Decreto Ministeriale, 31 May 1995, no. 292). Recently, a new official calvhoo vaccine, prepared with live attenuated *Brucella abortus* strain RB51, has been approved in the United States for use in a brucellosis eradication program. *B. abortus* RB51 is a lipopolysaccharide O-antigen-deficient mutant of virulent strain 2308 of *B. abortus* (8). RB51 has been documented to be protective in cattle and not to induce antibodies that interfere with brucellosis serological surveillance tests that mainly identify antibodies to lipopolysaccharide (5, 9). However, because of the absence of available serological tests for RB51, animals illegally vaccinated with this strain can elude detection in countries in which vaccination is not allowed. In addition, the diagnosis of a possible RB51 infection in humans exposed to this vaccine is also complicated (4). Previous studies have demonstrated that complement fixation (CF) tests using *B. abortus* RB51 as an antigen, previously deprived of anticomplementary activity due to its rough phenotype, are able specifically to detect cattle that have been experimentally vaccinated with RB51 (1). In addition to cattle, RB51-vaccinated sheep develop antibodies that can be de-

tected by CF tests using RB51 antigen, for a longer period than these tests can detect antibodies induced in cattle (2). The purpose of our study was to compare the suitability of antigens from *B. abortus* RB51 and hot saline extract (HSE), the latter now officially used as an antigen for detection of *B. ovis*-infected sheep, in detecting sheep experimentally vaccinated with *B. abortus* strain RB51 and sheep naturally infected with *B. ovis* or *Brucella melitensis* field strains.

MATERIALS AND METHODS.

***Brucella abortus* RB51 vaccine suspension.** For sheep vaccination, a suspension of *B. abortus* RB51 from a commercially available vaccine containing 5×10^9 CFU per ml was used. Strain RB51 was biochemically and serologically identified by the method of Schurig et al (8).

Animals and vaccination. For this study, six 5-month-old sheep from brucellosis-free herds were used and were kept in a concrete isolation room. After an acclimation period, four sheep were inoculated subcutaneously with 1 ml each of vaccine suspension as described above plus two booster vaccinations. The booster vaccinations were carried out at 110 and 234 days postvaccination (PVD). The remaining unvaccinated animals were used as untreated controls.

Collection of sera. Blood samples were collected for serological testing from vaccinated sheep and from controls before the vaccination (time 0) and at 7, 15, 30, 48, 76, and 110 PVD. Blood samples were also collected at 7, 15, 30, 36, 60, 100, and 124 days after the first booster dose and, finally, 12 days after the second booster dose. After collection, blood was allowed to clot at 4°C, it was centrifuged, and serum was stored at –20°C until used. To verify, the cross-reactivity against all antigens used in this study, eight serum samples were also collected from sheep microbiologically proven to be naturally infected with *B. melitensis* and *B. ovis*.

Reagents used in serological tests for brucellosis. For serological tests, the following standard antigens and antisera were used: HSE from *B. ovis* reference strain 63/290, standardized against the Office International des Epizooties first international standard *B. ovis* serum; *B. abortus* strain 99 inactivated antigen for CF and Rose Bengal Plate (RBP) tests, previously standardized against OIE second international standard *B. abortus* serum; a *B. abortus* RB51 inactivated suspension prepared as described below; OIE first international standard *B. ovis* serum, containing 1,000 IU per ml; OIE second international standard *B. abortus* serum, containing 1,000 IU per ml; and ovine anti-*B. abortus* RB51 serum experimentally produced in sheep vaccinated with *B. abortus* RB51.

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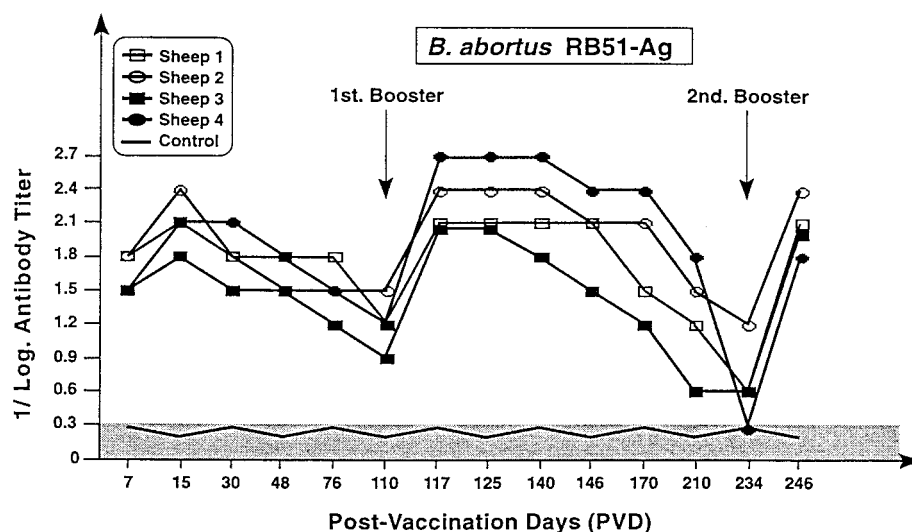


FIG. 1. Serum antibody responses of sheep following vaccination with *B. abortus* RB51 and boosters given at 110 and 234 PVD, as measured by RB51-based CF tests.

Preparation and standardization of antigen for *B. abortus* RB51 CF tests. Unlike smooth brucellae, the RB51 rough strain of *B. abortus* shows considerable anticomplementary activity. Thus, the RB51 strain used for CF testing was previously deprived of anticomplementary activity as follows: briefly, RB51 bacteria from a commercially available *B. abortus* vaccine RB-51 live culture were cultured for 48 h at 37°C on tryptose agar (Difco) supplemented with 5% bovine serum. After incubation, the bacteria were harvested with physiological saline (0.15 M NaCl [pH 7.2]) and washed twice by centrifugation at $6,000 \times g$. The suspension, containing 3×10^{10} CFU per ml as determined by plate counts, was divided into two aliquots and one was inactivated three times at 60°C for 1 h. Both aliquots were diluted twofold, and 25 μ l of each dilution was tested in a block titration assay against 25 μ l of negative bovine serum, previously inactivated at 58°C for 30 min. After incubation overnight at room temperature, each well was tested for the anticomplementary activity by the method of Alton et al. (3) with minor modifications (1, 2). Sensitivity of CF tests was evaluated by titrating inactivated and live antigens against the ovine anti-*B. abortus* RB51 serum. The specificity was evaluated by testing RB51 antigens against OIE second international standard *B. abortus* and OIE first international standard *B. ovis* sera and against pooled ovine serum from brucellosis-free herds. The RB51 antigens for CF tests were used at the lowest dilution of RB51 that showed complete absence of anticomplementary activity against 2 U of complement when incubated with bovine serum.

Serological analysis. All serum samples from RB51-vaccinated sheep, controls, and *B. melitensis* and *B. ovis* naturally infected sheep were tested for the presence of anti-*Brucella* antibodies by CF and RBP tests using conventional S-type *B. abortus* strain 99. Antibodies to *B. abortus* RB51 were measured by CF tests using inactivated RB51 and HSE as antigens.

RESULTS

Titration of RB51 antigens. The 1:32 dilution of both inactivated and live antigens proved to be the most sensitive dilution, enabling detection of *B. abortus* RB51-positive serum in the complete absence of anticomplementary activity against 2 U of complement. However, inactivated RB51 antigen showed 25% anticomplementary activity against 1 U of complement, while live antigen showed complete absence of this effect against 1 U of complement.

Sensitivity and specificity of RB51 antigens. Sensitivity test results for the RB51 antigens confirmed that both inactivated and live antigens are able to react against positive anti-RB51 ovine serum, showing a 1:64 titer. Regarding specificity, inac-

tivated RB51 antigen against OIE second international standard *B. abortus* and OIE first international standard *B. ovis* sera was titrated to 1:8 and 1:16, respectively. No reaction was observed against negative ovine serum. Live RB51 antigen against standard sera as above, was titrated to 1:16 and 1:32, respectively, while no reaction was detected against negative ovine serum. To minimize the risk for laboratory technicians, all CF tests were carried out by using inactivated RB51 antigen.

Serological evaluation. The results of CF tests performed on RB51-vaccinated sheep are shown in Fig. 1 and 2. As shown in Fig. 1, RB51 vaccination developed peak antibody titers at 15 PVD with high titers still present in all vaccinated sheep at 110 PVD when the first booster vaccination was carried out. The antibody responses following the first booster vaccination peaked at 7 days, and three out of four sheep showed still weak seropositivity at 234 PVD, when the second booster was given. The second booster induced high titers to RB51 as revealed by testing samples at 246 PVD. Following booster immunizations, some sheep had, a positive response to HSE antigen, although weaker than to RB51, that lasted about 30 days after the first booster. The second booster also induced a strong antibody response in some sheep, but only a weak response in others (Fig. 2). None of the RB51-vaccinated sheep developed antibodies detectable by *B. abortus* 99-based CF and RBP tests (data not shown). The unvaccinated sheep remained seronegative in all tests.

Cross-reactivity of sera from naturally *B. ovis* and *B. melitensis*-infected sheep. Sera from *B. ovis*-infected sheep, showing titers to HSE ranging from 1:4 to 1:128, did not react to *B. abortus* 99 antigen at a starting dilution of 1:2. When tested against RB51 antigen, *B. ovis*-infected sheep gave titers from 1:2 to 1:32, which were lower than those detected by HSE, as confirmed by testing the OIE anti-*B. ovis* serum against RB51 and HSE. The difference between RB51 and HSE titers was not significant ($P = 0.057$) (Fig. 3). Sera from

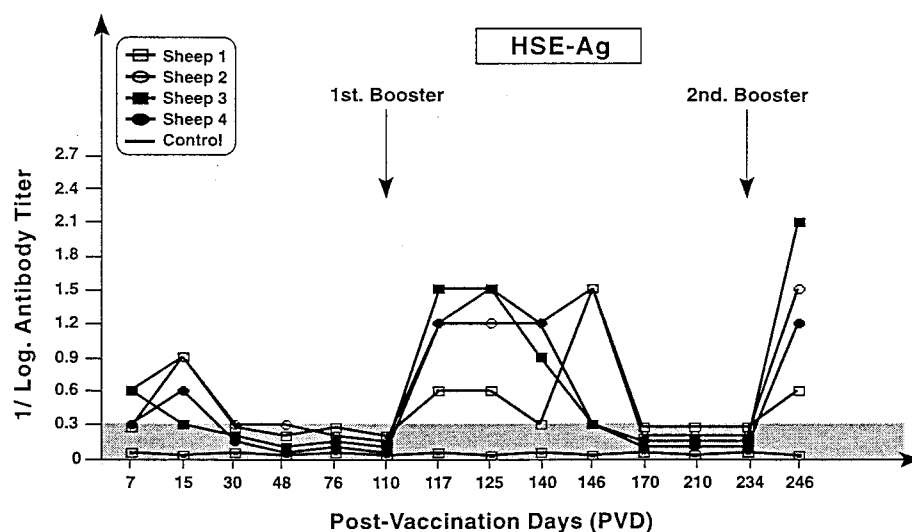


FIG. 2. Serum antibody responses of sheep following vaccination with *B. abortus* RB51 and boosters given at 110 and 234 PVD, as measured by HSE-based CF tests.

B. melitensis-infected sheep, showing titers to *B. abortus* 99 ranging from 1:4 to 1:64, did not react against RB51 or HSE. However, standard OIE anti-*B. abortus* serum containing 1,000 IU per ml showed titers of 1:8 in the RB51-based CF test, while no reaction was observed against HSE at the starting dilution (Fig. 4).

DISCUSSION

The results of this study confirmed that available serological surveillance tests for *B. abortus* 99 fail to detect seroconversion following *B. abortus* RB51 vaccination in sheep, thus supporting the results obtained in cattle (9) by using *B. abortus* 1119 smooth antigen as described previously (3). To measure antibody responses in RB51-vaccinated cattle, an experimental dot-blot assay with a gamma-irradiated suspension of RB51 as antigen has been performed (6, 7). Dot blot antibody titers obtained by testing sera from cattle vaccinated with 1.3×10^{10}

to 1.6×10^{10} CFU of RB51 were greater than those from unvaccinated cattle until 6 to 8 weeks postvaccination, while the results of our studies showed that cattle vaccinated at the same age with 10^{10} CFU of RB51 developed antibodies detectable by RB51-based CF tests until 15 weeks following vaccination (1). RB51-vaccinated sheep also developed antibodies to *B. abortus* RB51 that could be detected by CF tests for a more extended period than could antibodies induced in cattle. In fact, high titers were still present at 110 PVD when the first booster was given, as confirmed by previous studies (2). Following booster immunizations, these sera reacted positively against HSE, showing antibody responses weaker than against RB51. In addition, seropositivity to HSE remained for a very short period after the vaccination (30 PVD) and after the first booster (36 to 60 PVD). Sera from sheep naturally infected with *B. melitensis* seroconverted to the *B. abortus* 99 strain, as expected, while no reaction was observed against RB51 or

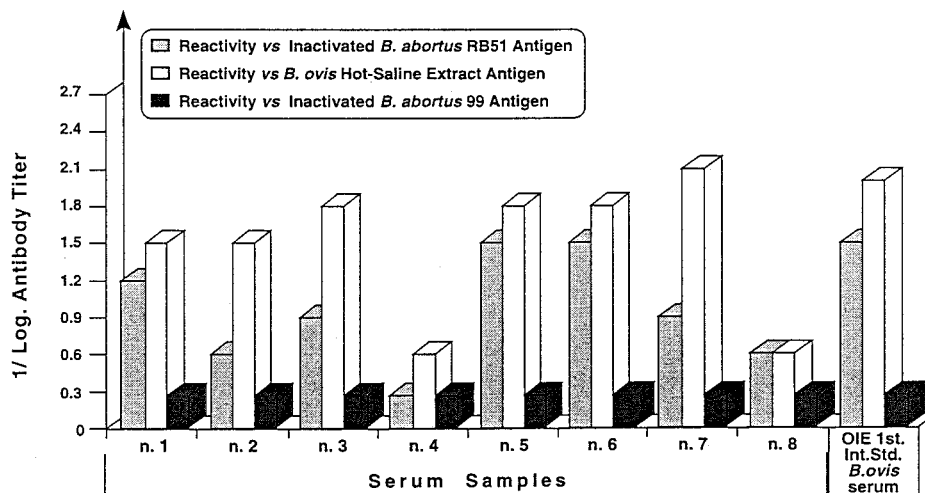


FIG. 3. Cross-reactivity of sera from eight *B. ovis*-infected sheep as measured by CF tests against different antigens.

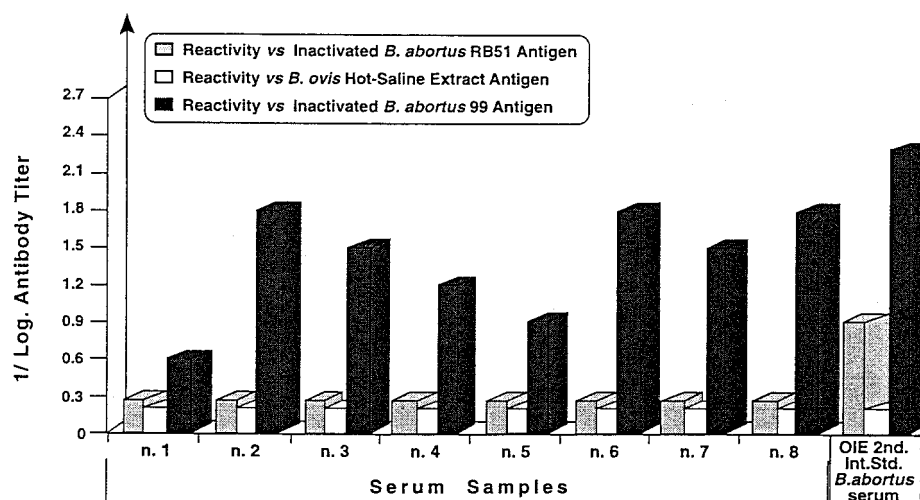


FIG. 4. Cross-reactivity of sera from eight *B. melitensis*-infected sheep as measured by CF tests against different antigens.

HSE antigens. The positive reaction obtained by testing *B. abortus* international standard serum to RB51 (titer, 1:8) was significantly lower compared with that obtained against *B. abortus* 99 antigen, thus enabling a distinction between RB51-vaccinated and S-type *Brucella*-infected sheep. Sera from sheep naturally infected with *B. ovis* reacted against RB51 antigen in CF tests giving titers lower than those obtained by using the official HSE antigen. However, because of the cross-reactivity, the identification of naturally *B. ovis*-infected sheep, especially in areas with a high *B. ovis* infection prevalence, should be microbiologically confirmed. In conclusion, the results of this study indicated that, in comparison with HSE antigen, *B. abortus* RB51 previously deprived of anticomplementary activity proved to be the most useful antigen to serologically detect RB51-vaccinated sheep, thus suggesting the use of an RB51-based CF test in brucellosis eradication programs to identify abuse of RB51 vaccine in sheep. To confirm the suitability of this test, studies are in progress in our laboratory under field conditions to evaluate the efficacy of this CF test in detecting antibody responses of cattle and sheep from countries where RB51 is currently used as a vaccine for brucellosis.

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